Isolation of bacteria from positive blood cultures by filtration to facilitate rapid identification and antimicrobial susceptibility testing in sepsis diagnostics

By
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Abstract

Sepsis is a time-sensitive condition in which fast and accurate diagnosis is beneficial to patients. The current gold standard in diagnosing sepsis requires plating a sample from a positive blood culture in order to isolate bacteria for further testing. This sub-cultivation step requires on average 18 hours of incubation to grow a sufficient number of bacteria colonies, therefore introducing a delay to perform downstream tests. However, the replacement of this step with a faster method will allow clinicians to identify the responsible pathogen and administer the appropriate antibiotics sooner. To reduce time to results, a method based upon tangential flow filtration was developed to isolate viable bacteria from a positive blood culture in a matter of minutes. This method produces a bacteria suspension that excludes blood cells and analytes, allowing the sample to be used in subsequent identification and antimicrobial susceptibility testing. By eliminating the subcultivation step, patients can receive optimized treatment earlier, which is correlated with positive clinical outcomes.

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Introduction

Sepsis

Sepsis, a systemic inflammatory response to infection, causes more than 751,000 hospitalizations and 215,000 deaths in the United States annually,[1] with the number of cases increasing by 13.7% each year.[2] Severe sepsis is reported in 2% of patients admitted to the hospital, of which half are treated in the ICU, representing 10% of all ICU admissions[3] and costing $17 billion in annual health care expenses.

Signs of a systemic inflammatory response, such as faster heart beat (tachycardia) or an elevated white blood cell count, can occur in many infectious and noninfectious conditions, so they may not help to differentiate sepsis from other conditions. One of the main defining characteristics of sepsis is the presence of microorganisms in the blood, in addition to other clinical symptoms for Systemic Inflammatory Response Syndrome (SIRS), as listed in Table 1.[4] Sepsis can evolve into severe sepsis when it is complicated by acute organ dysfunction and further progress to septic shock when sepsis is accompanied by either persistent hypotension or hyper-lactatemia.[5,6] A summary of the stages of sepsis is outlined in Table 1 below. Severe sepsis can be acquired from both community and health care-associated infections, with pneumonia as the most common cause, accounting for about half of all cases, followed by intra-abdominal and urinary tract infections.[3] The most common isolates responsible for infection, such as *Escherichia coli* and *Staphylococcus aureus*, and their prevalence are shown in Figure 1.

Table 1. Systemic Inflammatory Response Syndrome (SIRS) and sepsis stages[7]

Stage	Clinical symptoms	Value
SIRS (≥2 symptoms)	Temperature	<36 °C or >38 °C
	Heart rate	>90 beats/minute
	Respiratory rate	>20 breaths/minute or $PaCO_2$<32 mmHg
	White blood cell count	>12,000 cells/mm^3 or < than 4,000 cells/mm^3
Sepsis	SIRS with proven infection	
Severe sepsis	Sepsis with organ dysfunction hypoperfusion and/or hypotension	
Septic shock	Severe sepsis, hypoperfusion and/or hypotension despite adequate fluid resuscitation	

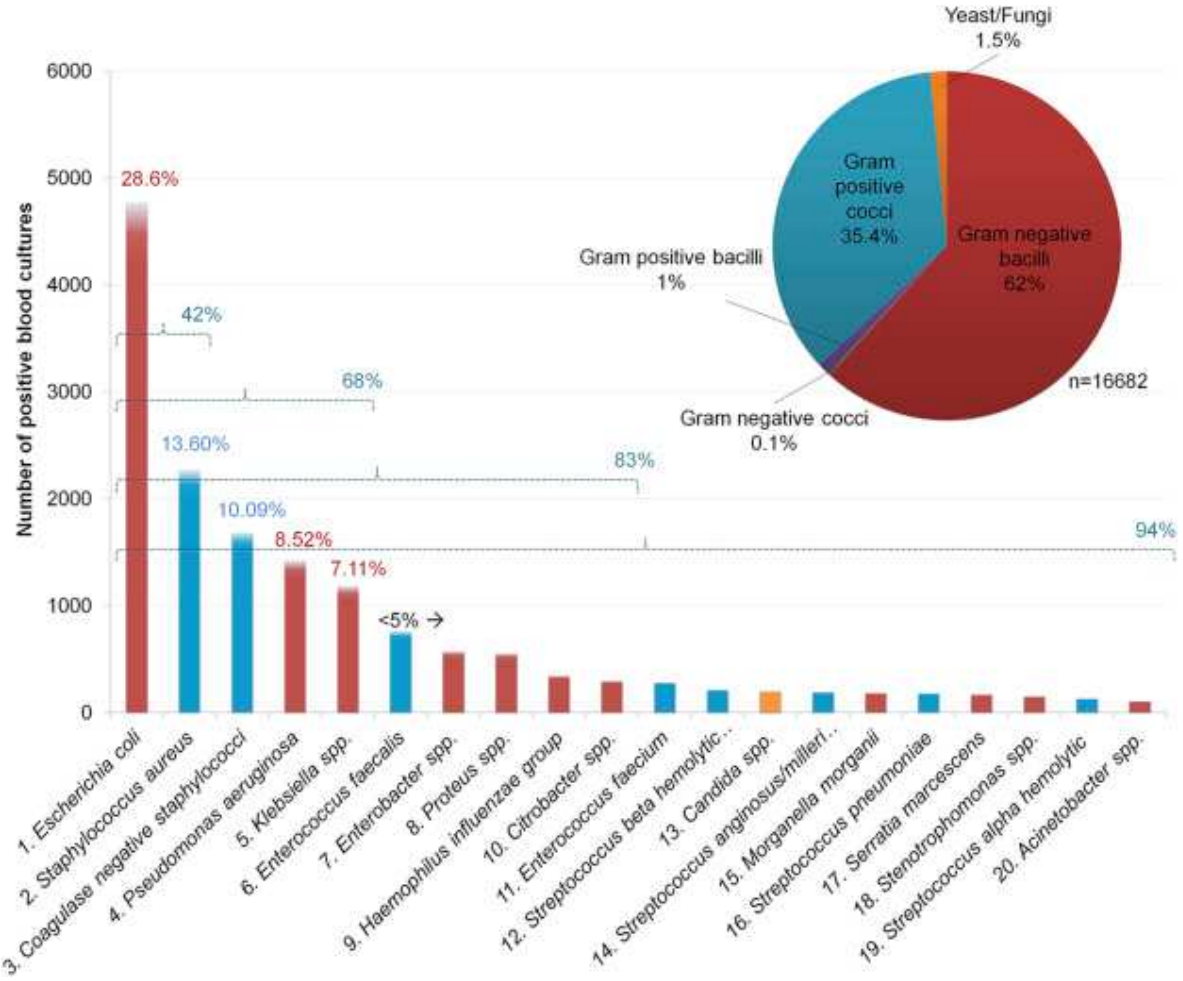


Figure 1. Top 20 microbes identified from positive blood cultures during 2013 in a tertiary-care university hospital. The pie chart represents the distribution per morphotype of all the microorganisms (total 16,682 identifications).[8]

Sepsis diagnostics: state of the art

Once a patient is suspected of having sepsis, the clinician is responsible for forming a plausible diagnosis, obtaining blood cultures, and administering empirical antimicrobial therapy. A rapid assessment can be made based on the patient's history and clinical symptoms presented. Clinicians will administer a set of broad spectrum antibiotics prior to the precise diagnosis since early appropriate antibiotics are associated with improved survival. The choice of initial empirical antibiotic therapy depends on the likely source of infection, the most likely pathogens involved and related susceptibility profiles.[6] Then, investigations to confirm the presence, source and severity of the infection need to be performed to refine diagnosis and treatment.

Blood cultures are one of the most important clinical laboratory tests performed as the gold standard for diagnosing bloodstream infections (BSIs). The number of microbes present during BSIs range from 1 to 10 colony forming units (CFU) per mL of blood, and these bacteria are allowed to grow in cultivation media.[8] Once the blood culture bottle goes "positive", indicating that bacteria growth is detected, Gram staining is performed which determines the morphotype of the bacteria (Gram-positive or Gram-negative) and allows for the first adjustment of antibiotic therapy. In some cases, the blood culture may be polymicrobial instead of monomicrobial, such that both Gram-negative and Gram-positive organisms are present. Polymicrobial BSIs encompass approximately 6 – 32% of all BSI episodes.[9]

Adjustment of antibiotic therapy is necessary to reduce the potential for antibiotic resistance and other side effects, such as increased risk for mortality, due to inappropriate antimicrobial treatment.[10] Plating a sample of this positive blood culture (PBC), otherwise known as the subcultivation step, is then needed to isolate the bacteria for

identification (ID) and antimicrobial susceptibility testing (AST). Isolation of the bacteria from PBC is needed for faster ID using matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS). ID straight from blood culture is possible but yields spectra of lower quality than those from colonies, sometimes making it difficult to differentiate between closely related species.[11,12] Excellent spectra are obtained when a minimum of 10^6 CFU are spotted onto the MALDI target plate, which is a concentration easily obtained from culture plates but at the threshold of what is normally present in a PBC bottle. Inadequate spectra are usually the result of an insufficient amount of bacteria on the target plate.[13] Furthermore, bacteria isolates are needed to accurately inoculate a known bacteria concentration into AST systems. AST systems such as the BD Phoenix system will notify the clinician what drugs (and at what dose) can be used to treat the patient. Identification of the bacteria is needed to interpret the antimicrobial profile results so that the clinician can make the appropriate changes and further deescalate antibiotic therapy.

Currently, it takes up to 2 days or even longer for a septic patient to be thoroughly tested and diagnosed. Figure 2 outlines the basic workflow starting from when a patient presents clinical symptoms of sepsis to the final stages when an accurate and complete diagnosis is made. The median time to detect a positive blood culture is approximately 12 hours but may be longer or shorter. [14] One of the most time consuming steps is the 12- to 24-hour incubation period required for plating to obtain enough isolated colonies from a PBC for characterization. [8] The following results are obtained within an hour for ID and in 4-16 hours for AST. However, by that time the results are usually too late to influence vital treatment decisions.[15,16]

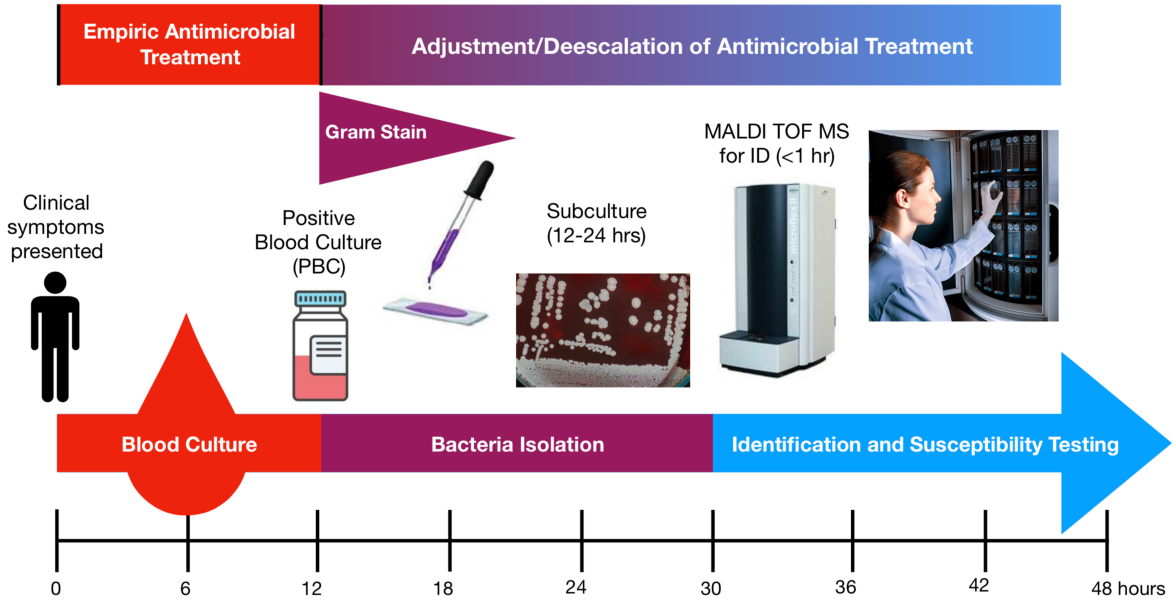


Figure 2. General work flow in diagnosing a septic patient.

Although subcultivation is the most common way to process a PBC for further testing, there are other approaches that do not require this plating step. Two main alternatives include molecular-based methods which allow for ID directly from a PBC and enrichment/purification methods that can obtain a bacteria pellet from a PBC.[8] These approaches are mainly useful for ID because they do not enable reliable AST. Molecular methods are generally faster than phenotypic approaches because they can be used directly on PBCs. These nucleic-acid-based methods significantly reduce the turn-around time to results to 1.5 – 4 hours and are usually highly sensitive and specific, but large-scale usage of these technologies in the clinic is hindered by the expensive costs, substantial hands-on time, and lesser sensitivity and specificity compared to blood cultures.[17] Furthermore, molecular diagnostics can only detect a limited number of drug resistance markers and genes, and do not provide as comprehensive results compared to

phenotypic AST. Another subculture independent approach is to obtain a microbial pellet from a blood culture, such as through the Bruker's MALDI Sepsityper. This method can be used with MALDI-TOF MS and provide ID results in less than an hour, but requires lysing of cells and multiple buffer washing steps with centrifugation. Therefore, this labor-intensive process will hinder their implementation into laboratories with limited human resources. In addition, using the produced bacteria pellet with AST systems is suboptimal because the lysing and centrifugation steps decrease the viability of the bacteria of interest, leading to discrepancies in results compared to conventional approaches.[16] Stressed organisms may respond differently to antibiotics compared with those organisms that have been inoculated into the AST panel from subculture.[18]

Clinical need

To address increasing rates of drug resistance, clinicians are compelled to administer empiric broad-spectrum antibiotics to patients with presumed bacterial infection as soon as possible. However, the initial antimicrobial treatment used is an important determining factor of survival in patients with sepsis; inappropriate initial antibiotic treatment can increase mortality. Administration of effective antibiotics within the first hour of documented hypotension was associated with a survival rate of 79.9%. Furthermore, each hour of delay in antimicrobial administration in the following 6 hours was associated with an average decrease in survival by 7.6%.[19] More importantly, de-escalation of initial broad-spectrum therapy may prevent the emergence of resistant organisms and minimize the risk of drug toxicity.[16] For those reasons, the time required to identify the bacteria and receive AST results has a critical influence on subsequent

therapy. In one study, reducing the turnaround time for results by almost a full day allowed for earlier adjustment of targeted antimicrobial therapy which correlated with positive clinical outcomes such as decreased length of stay, decreased mortality, and reduced health care expenditures.[19,20] The important relationship between prompt administration of optimized antibiotic therapy and improved patient outcome highlights the advantage of shorter time to results. This project proposes to decrease time to results by addressing the subcultivation step which is time spent by waiting for pure bacteria isolates to grow. Replacing this conventional method with a quicker alternative to provide bacteria isolates will enable faster ID and AST.

Project aim

The primary goal of this project is to develop a rapid method to isolate viable bacteria from a mono-microbial PBC in order to replace the time-consuming subcultivation step. Replacing this conventional method, which takes on average 18 hours, with a method that provides a bacterial isolate on the order of minutes, allows for reduced time to results. Consequently, clinicians can accurately diagnose septic patients and administer targeted antimicrobial therapy in a timely manner, ultimately improving patient outcome.

The final output sample provided by this new PBC processing method needs to integrate with pre-existing ID and AST systems. To allow for accurate results, the isolated bacteria need to be relatively pure so that any blood cells or analytes that can interfere with subsequent testing are excluded. Also, the bacteria need to be viable and uncompromised in order to yield accurate AST results. Lastly, a final bacteria suspension ranging from 10^8 total CFU in 100 µL to 10^9 total CFU in 1 mL is required to provide sufficient bacteria sample to be tested in both ID and AST systems. For this project, the PBCs of interest are limited to mono-microbial PBCs because the separation of different bacterial species from each other introduces additional difficulties that can be addressed further on. The design requirements for the proposed process are summarized in Table 2 below.

Table 2: Design constraints and targets for PBC processing method

Specification	**Target**
Input	PBC—monomicrobial, any media type, any bacterial species
Output	Viable bacteria isolates (at least 10^8 CFU in 100 μL or 10^9 in 1 mL) with minimal blood background contributing to optical density
Compatibility	Interfaces with both ID and AST systems
	Allows for Gram staining of bacteria suspension
Time	Less than 15 minutes to isolate bacteria from PBC
Cost	Low-cost disposable

Approach

An alternative method based on filtration is proposed to isolate viable bacteria from a monomicrobial PBC as a means to replace the time-consuming and cumbersome process of subcultivation. Filtration can be used to isolate the bacteria of interest from other components in the PBC such as blood cells, salts, and antibiotics. With filtration, different methods and processing parameters need to be considered to optimize efficiency and maximize bacteria recovery to achieve the required amount for downstream testing. The hydrodynamic forces that the blood components and bacteria experience during filtration can also influence how readily separation occurs.

Filtration methods

Filtration allows for the separation of solutes so that molecules and particles of different sizes or molecular weights can be sorted based on the membrane pore diameter.

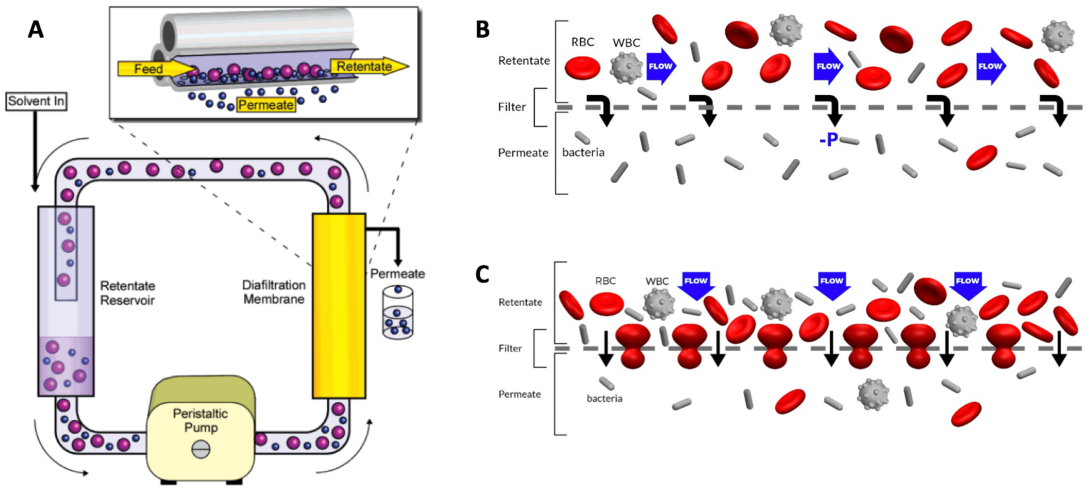


Figure 3: (A) Diagram of an example TFF system[21] (B) TFF with PBC components in flow along the filter membrane (C) Dead-end filtration with PBC components in flow.

In tangential flow filtration (TFF), also known as cross-flow filtration, a pump drives the feed fluid from the retentate reservoir along the membrane, allowing the fluid to recirculate, while the same or different pump produces a transmembrane pressure (TMP) which draws the permeate across the membrane (Figure 3A). Components larger than the pore size are retained in the feed solution as the retentate while solutes smaller than the pore diameter will be collected in the permeate.[22] As seen in Figure 3B, the feed suspension in TFF flows parallel to the filter membrane and perpendicular to the flow of filtrate. [23] The TFF system also allows for the addition of solvent during filtration to dilute the retentate as permeate is removed (Figure 3A).

Another popular means of filtration is dead-end filtration, which is typically used as a single-pass method with a constant system pressure across the membrane to yield permeate. In this case, as seen in Figure 3C, the feed solution flows perpendicular to the filter membrane and the permeate flows straight through the membrane's pores, resulting in a higher likelihood of particles clogging the pores. Therefore, dead-end filtration is an appropriate means to concentrate or separate particles in large sample volumes, but may not be as suitable for purification applications that require washing. [24]

For both tangential-flow and dead-end filtration, separation of molecules or particles from fluid poses a problem to the integrity of the filtration membrane over time due to fouling. The accumulation of particles on the membrane surface can be limited but unavoidable.[25] In this application, a PBC containing blood cells, bacteria, cell debris, and other media components is filtered through a membrane. Although the hematocrit level is reduced five-fold compared to whole blood, there is a substantial number of blood cells left (about 10^8 cells/mL) and the bacteria have grown to reach a concentration of 10^7 –

10^9 CFU/mL. For TFF, as the fluid recirculates on the retentate side, the solutes are brought to the membrane surface, forming a layer of rejected solutes, which increase the resistance to filtration. Also, the TMP will further pack and condense this layer so that the membrane becomes increasingly less permeable as the pores are narrowed or blocked, and also less efficient in separating small solutes from larger ones in the retentate. Therefore, it is necessary to circulate the retentate at high enough speeds to generate a high enough shear rate to increase back transport away from the membrane, reduce concentration polarization, and limit cake formation on the membrane surface. By increasing the cross-flow velocity and the shear rate, the particles are carried away from the membrane and brought back to the bulk feed solution by tangential flow, forming a thinner cake layer.[26] However, in unstirred dead-end filtration, the accumulation of solutes on the surface or inside the pores of the membrane will quickly decrease the permeation flux. Therefore, this process is only practical for microfiltration of low-concentration fluids such as surface water. For TFF, continuous recirculation of feed and optimization of both the flow rate and TMP are able to control fouling. On the other hand, in dead end filtration, a batch filtration method, the system needs to be stopped for backwashing in order to remove any caking that may have occurred.[22]

Membrane filtration can be operated in dead-end or cross-flow modes. The choice depends on the importance of productivity compared to operational costs. For solutions with high fouling potential, cross-flow filtration is necessary, but for feed solutions that are less likely to foul, dead-end filtration is usually more economical as a batch process. Since the retained particles continuously accumulate on the membrane surface during filtration, periodic backwashing is required to remove deposited particles. [27] For the

application of interest, TFF is a continuous process that is preferable for its ability to control fouling using the system parameters. Furthermore, the reduction in caking provided by TFF will help to maintain permeate flux, improve efficiency, and maximize bacteria recovery due to minimized adhesion of bacteria to the membrane.

Hydrodynamic forces acting on blood components

Blood is composed of RBCs, WBCs, platelets, and plasma, with the ratio of WBCs against RBCs to be 1:600-1,000. RBCs are biconcave disks and are known to be extremely deformable. The viscosity ratio between the inner fluid of the RBC and the carrier fluid can partly explain the behavior of RBCs in their environment. [28] At small shear rates and at rest, RBCs have a biconcave discoid shape; but at high shear rates, the cell shape adopts to the parabolic flow field and the cells become parachutes. [29–31] In Poiseuille flow with a parabolic flow profile, deformable RBCs will migrate away from the wall towards the center of the channel due to cell-wall interactions. This hydrodynamic lift force experienced by the RBCs is known as the Fåhræus-Lindqvist effect which also drives the WBCs and blood platelets to migrate towards the walls, due to a phenomenon known as margination.

The hydrodynamic lift force is dependent on: shape of the particle, deformability of particle, density difference between the particle and fluid, and shear stress. [28,32] The typical size distribution of blood components and bacteria is outlined in Table 3. Since both RBCs and platelets are discoid shaped and have a soft membrane, their size difference affects their lift velocity; the larger RBCs experience a greater lift force and will migrate closer to the center of the channel. The stiffer and more rigid WBCs do not experience lift force, although they can rotate. The bacteria, which are about the same

size as the blood platelets, are then separated by their deformability, in which deformable platelets will be lifted and rigid bacteria will experience no lift force. [32] Knowledge of the interactions between cells and how blood components flow through the channels can be exploited for high efficiency filtration. As seen in Figure 4, if Poiseuille flow exists, the deflection of RBCs away from the walls and presence of bacteria closer to the membrane can help increase filtration efficiency.

Table 3: Size of RBCs, WBCs, platelets, and bacteria (e.g. *E. coli*) [32,33]

	RBCs	WBCs	Platelets	Bacteria
Diameter (µm)	7-8	12-15	2-4	2
Thickness (µm)	~2	12-15	~1	~1

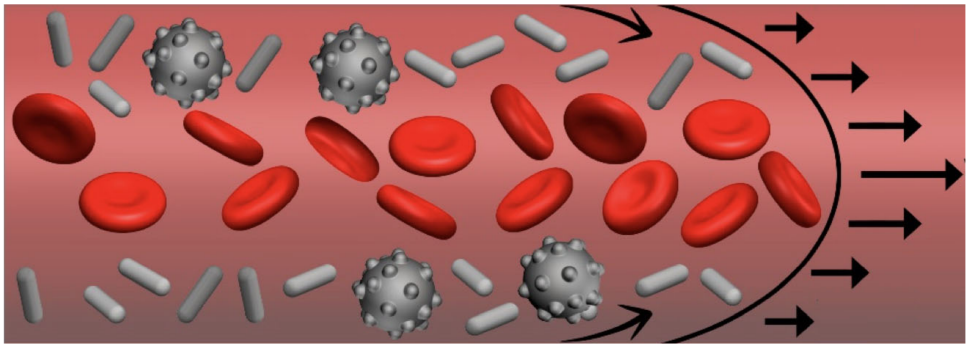


Figure 4: PBC components flowing in a channel under Poiseuille flow

Biofouling

As mentioned earlier, membrane fouling is a significant concern during filtration and understanding how to minimize it is essential. Membrane fouling is the undesirable deposition of retained particles, colloids, macromolecules and salts at the membrane surface or inside the membrane's pores. Biofouling describes instances in which biologically active organisms, such as microorganisms, and their excreted extracellular

biopolymers are involved. The consequences of biofilm formation on membranes include: increased membrane resistance, decreased permeate production, increase of drag resistance to tangential flow, increased differential pressure, and concentrated populations of microorganisms on membrane surfaces. [34,35] Membrane fouling can be somewhat controlled by the selection of an appropriate membrane and adjustment of the operating conditions, but very often these are not enough to address all the sources of biofouling.

As the bacteria approaches the surface of the membrane, there are several forces at work between the bacterial cell wall and the membrane. These forces include hydraulic forces, electrostatic forces, mechanical attachment with polymeric surface structures and hydrophobic interactions. Initially, the cell is only reversibly attached and can be removed by a change in shear force. However, after a certain amount of time, the bonds between the cell and membrane surface become more permanent. Some bacteria have surface appendages such as pili, or fimbriae, which anchor onto the membrane structure. The extra-cellular polymeric substance (EPS) secreted by the bacteria helps to form a polymer bridge between the cell and surface which becomes more stable over time. [36]

Membrane properties to address biofouling

For the TFF system developed for this project, characteristics of the filter membrane can be modified to meet performance criteria and reduce biofouling.

One main property that can be considered to reduce bacteria adhesion is the hydrophobicity of the membrane. Fouling occurs more readily on hydrophobic surfaces because there are minimal hydrogen bonding interactions in the boundary layer between the membrane surface and water. Water molecules are repelled away from hydrophobic surfaces due to entropy, so foulant molecules can adsorb onto the surface more easily.

However, hydrophilic surfaces possess a high surface tension, allowing for the formation of hydrogen bonds with the surrounding water molecules and the construction of a thin water boundary between the membrane and bulk solution. Therefore, hydrophilic membranes are less susceptible to fouling with organic substances and microorganisms due to decreased interaction between the foulant and the membrane surface.[36]

Membrane charge is an important factor to consider especially when foulants are charged. If the foulant and surface have similar charge, electrostatic repulsion forces between them will prevent the foulants from depositing onto the membrane. Bacterial surfaces are usually comprised of phospholipids, lipopolysaccharides, polysaccharides and proteins, which are all charged at physiological pH. They usually carry negative charges and thus negatively charged membrane surfaces are less prone to bio-adhesion. However, some studies have shown that there is increased bacterial adhesion to surfaces with increasing negative zeta potential due to the formation of a conditioning layer. Cations are readily adsorbed onto the surface, and can act as bridges between the membrane surface and the cell. Despite this fact, there is a much steeper increase in fouling for surfaces with a positive zeta potential compared to those with a negative zeta potential. There is also some research that suggests that the interfacial electrostatic repulsion of the material extends for a small distance compared to the typical bacteria pili that extend 500-1000 nm from the bacterial cell surface, so bacteria could potentially overcome this extent of electrostatic repulsion. [37]

Although trying to mitigate biofouling by membrane modification is beyond the scope of this project, it is an important factor to investigate as the project progresses. In addition to the surface membrane properties, membrane fouling also largely depends on

the concentration of bacteria, nutrients in the feed solution, and the hydrodynamic forces within the system.[34] Optimization of operation conditions and feed solution along with targeted modification of surface properties should be able to minimize membrane biofouling.

Track-etched membranes

Polycarbonate track-etched membranes (TEMs) were chosen for their well-controlled pore size and cylindrical holes. These qualities are preferable compared to other membrane types such as polytetrafluoroethylene and nylon which possess irregular tortuous paths for permeate to follow. [38] These polycarbonate membranes also have low non-specific binding and are biologically inert, which can potentially help reduce membrane fouling. The TEM starts out as a roll of plastic film, which is then exposed to charged particles from a nuclear reactor, leaving behind latent tracks. Next, the film is treated with a caustic etchant which preferentially targets the tracks to form pores. A unique property of TEMs is that the pore density and size can be modified independently of each other as they are controlled by exposure time, concentration of the etchant, and temperature. The pore diameter can range from 10 nanometers to tens of microns and the pore density can vary from 1 to 10^{10} cm^{-2}. Polycarbonate TEMs have a lower wettability compared to PET membranes, which is why a wetting agent, polyvinylpyrrolidone, is needed to increase its hydrophilicity and allow this material to be used with aqueous solutions. [39]

Methods

Test Samples

Blood culture bottles (BACTEC Standard Aerobic culture vials) were spiked with 10 mL of bagged human blood and 10-100 colony forming units (CFU) of the bacteria of interest (1 strain per bottle). The appropriate number of bacteria was inoculated into the blood culture bottle to reflect the expected concentration of bacteria in a patient's blood. After inoculation, the bottles were loaded into a BACTEC FX instrument and allowed to incubate.

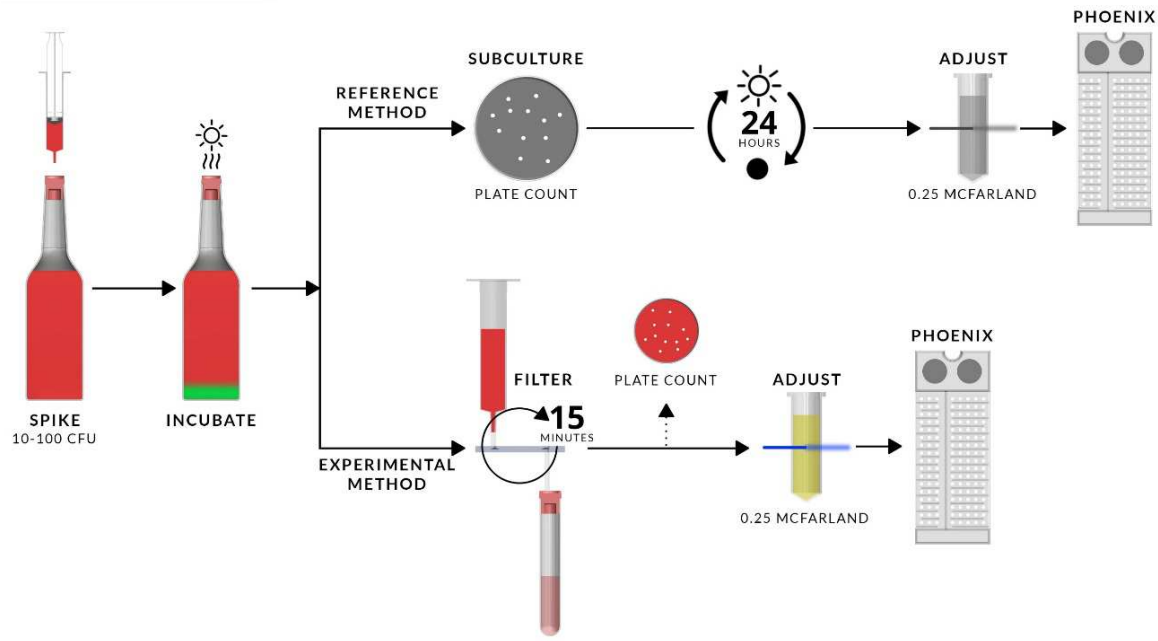


Figure 5. Experimental Plan after creation of a PBC test sample. In the reference method, a sample of PBC was plated to grow bacteria isolates. In the experimental method, the PBC was filtered using the new PBC processing approach to yield an isolated bacterial suspension. A 0.25 McFarland suspension of the isolated bacteria samples was then inoculated into a Phoenix panel for AST. Plate counts were performed in parallel in the experimental method to calculate the bacteria recovery after filtration.

Plate counts

Once the bottle was considered positive by the BACTEC instrument, it was removed and the initial bacteria concentration in the PBC was calculated by creating serial dilutions and inoculating TSA II 5% sheep blood plates with the sample. In the experimental method, the output bacteria sample after filtration was serially diluted and plated. Once the organisms had grown on the plates overnight, plate counts were performed to calculate the recovery.

Antimicrobial Susceptibility Testing

The bacteria samples isolated from two different methods were tested using the BD Phoenix AST system. The system determined the susceptibility of the bacteria to various drugs and determined the minimum inhibitory concentration (MIC), the lowest concentration of drug that prevents visible growth of a bacteria. Organism identification was used in the interpretation of the MIC values of each antimicrobial agent producing Susceptible, Intermediate, or Resistant (SIR) result classifications.

The Phoenix AST panel has growth control wells and wells with varying concentrations of antimicrobial agents. Prior to inoculating the AST panel, a bacteria suspension of a known concentration was made. The bacteria suspension, isolated from either subcultivation or filtration, was measured for its turbidity, which is the cloudiness of the sample. The optical density of the bacteria suspension could be compared to a McFarland standard, a turbidity standard, which allows for estimation of organism concentrations. A nephelometer was used to measure the scattered light from the particles in suspension and determine the McFarland equivalent. The isolated bacteria were added to a Phoenix ID tube (2.2 mL fill volume) to achieve a 0.25-0.30 McFarland suspension,

and then 25μL of that suspension was added to AST broth for AST panel inoculation. In this case, 0.25 McFarland is approximately 7.5 x 10^7 CFU/mL (based off of *E. coli*, ATCC 25922).

The panels were inoculated with this standardized organism suspension and the system read panels every 20 minutes to determine bacteria growth. The AST results of the isolated bacteria from the two different methods, the reference method using subcultivation and the new PBC processing step, were compared. The experimental plan is summarized in Figure 5.

Bacteria Identification

Bruker's MALDI Biotyper Microbial Identification system allows for species level identification of bacteria within a few minutes and is based on MALDI-TOF MS. For the preliminary MALDI study, only the output samples from the experimental filtration method were tested. The standard protocol for MALDI was followed with two different methods of sample preparation. The first method, straight testing, was done by spotting 1 μL of the filtered output sample directly onto the target plate. The second method, simple dilution, had 5 μL of the filtered output diluted with 5 μL of sterile deionized water, and 1 μL of the diluted sample was spotted onto the plate. Ten organisms were tested in duplicate (2 spots per processed sample).

Results

Passage of blood cells

For initial samples processed using the TFF system, blood cells were observed in the output sample after filtration, as seen by a pink tint in the final bacteria suspension. This finding, along with confirmation under the microscope, indicated that the blood cells in the PBC were not completely excluded from the bacteria under the current system. To quantitatively measure how many blood cells were eliminated with the bacteria into the permeate, a negative blood culture (NBC) was filtered under standard processing conditions with TEMs of different pore sizes. A NBC was created by adding 10 mL of bagged human blood to a BACTEC Standard Aerobic culture bottle to mimic the concentration of blood cells in a typical blood culture. Aliquots of 5 mL were removed from this NBC and filtered through filter membranes with pore sizes of 3, 2, and 1 µm in diameter. The standard processing parameters were used with a flow rate of 20 mL/min and a vacuum of –70 kPa. The permeate sample was diluted appropriately to improve ease of counting cells using a hemocytometer.

With the 3 µm pore size, about 10% of the original number of RBCs in the NBC, corresponding to 5.63×10^8 total RBCs, were able to pass through the membrane. As the pore size decreased by 1 µm in diameter, about 10 times less RBCs were able to be extracted into the permeate each time (Figure 6A). As seen in both Figures 6 B and C, there are a number of pores on the membrane that overlap each other, resulting in larger pores that may allow unexpected particles to pass through the membrane. Figure 6C shows that the RBCs at rest are much larger than the 2 µm pores but are still able to pass through the pores.

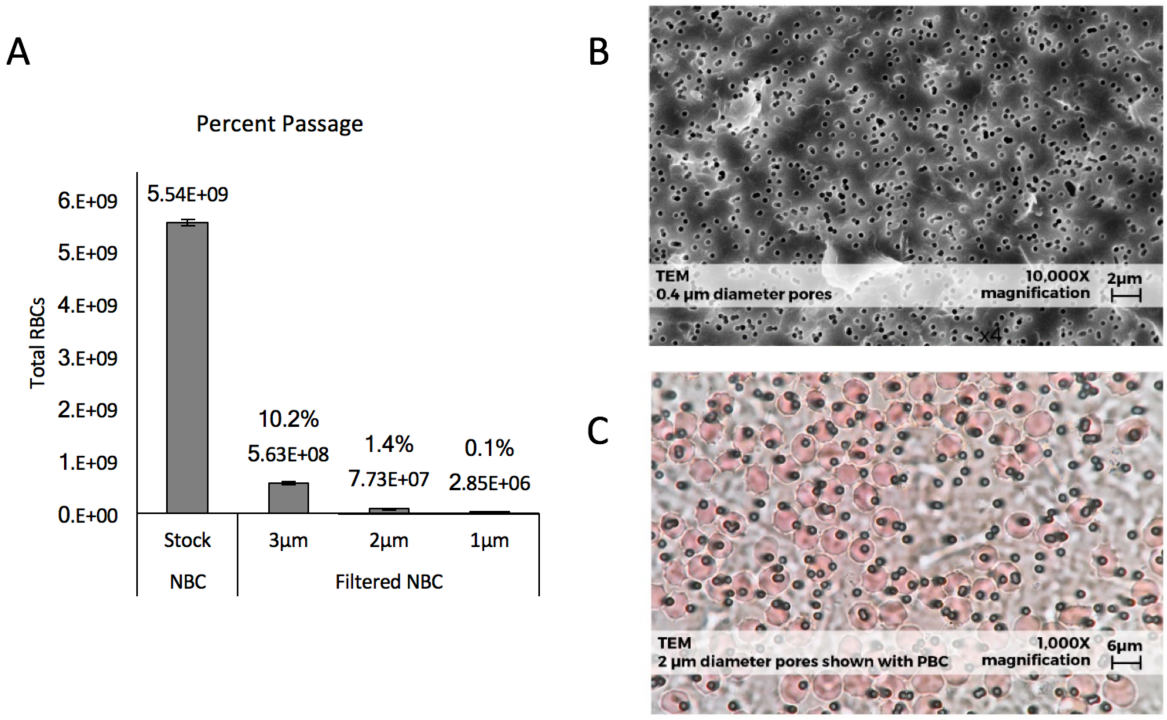


Figure 6. (A) Percent passage of blood cells through membranes of pore sizes 3, 2, and 1 µm in diameter. (B) Image of TEM with 0.4 µm pore size. (C) Image of a PBC on a 2 µm membrane to compare size of blood cells with pore size.

The presence of RBCs in the final bacteria output sample poses a problem in downstream processing, especially for accurate inoculation into an AST system. Since blood cells have their own optical densities, they could affect the nephelometer turbidity measurement and therefore the accuracy in creating a suspension with a known concentration of bacteria. However, lysing the blood cells was suspected to reduce the optical density and was tested using saponin, a lytic agent. To evaluate this prediction, the turbidity of a suspension of blood cells in Standard Aerobic media was measured. A suspension reflecting the number of blood cells that pass through a 2 µm filter (as indicated by Figure 6) was created by diluting a NBC to about 7.7 x 10^7 cells/mL. BD lytic media contains 0.26% w/v of saponin, which is a concentration that is able to lyse the blood cells without lysing the bacteria of interest. Different amounts of lytic media

were added to vary the ratio of standard media to lytic media while maintaining a constant concentration of blood cells and constant total volume. Turbidity measurements using a nephelometer with a McFarland standard were obtained every two seconds to see the change in turbidity over time. As seen in Figure 7, with increasing amounts of lytic media, the turbidity of the sample decreased to a minimum faster. Lytic media itself has an optical density of about 0.2 McFarland which can help explain why the measured turbidity in this experiment does not decrease to 0 for all tested conditions. However, the most important observation is that there is a steep decrease in turbidity after saponin is added to the blood suspension.

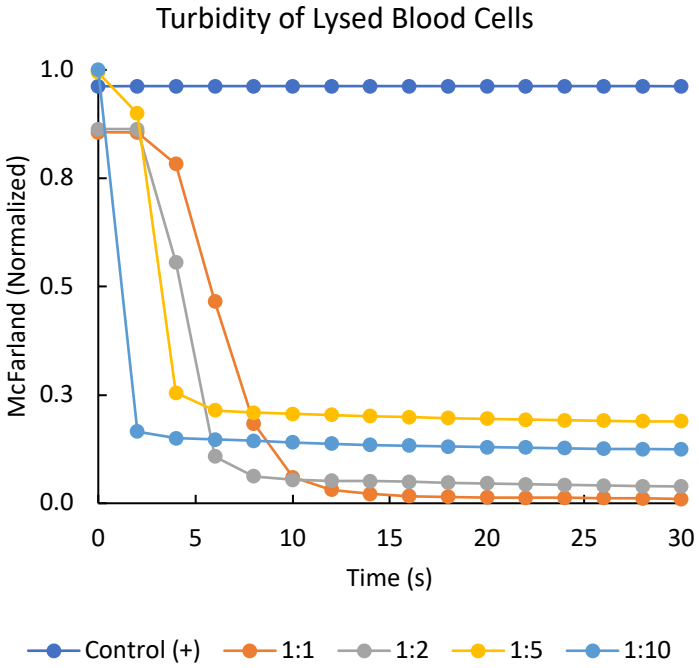


Figure 7. Turbidity of blood cell suspensions after addition of BD lytic media at different standard to lytic media ratios. The positive control was a blood cell suspension of approximately 7.7 x 10^7 cells/mL.

Bacteria recovery and loss

The filtration process evolved early on in the project, taking into consideration different factors that would affect the efficiency and efficacy of the filtration system. Initially, the feed PBC solution was directly filtered through the membrane until a minimum amount of feed solution was remaining. Then, the addition of saline solution to the feed solution during filtration was implemented to allow for longer circulation of bacteria in the TFF system and dilution of the sample to promote hydrodynamic lift; this additional step was believed to allow for greater recovery of bacteria. Later on, the inclusion of a lysing step was needed to reduce the contribution of blood cells to the turbidity measurements used for the Phoenix AST system.

A large scale study (n=83 bacterial strains) was performed for both Gram-negative and Gram-positive bacteria to determine the recovery efficiency of the new PBC processing method. The PBC aliquots were processed through the filtration system with a volumetric flow rate of 20 mL/min and TMP of -70 kPa.

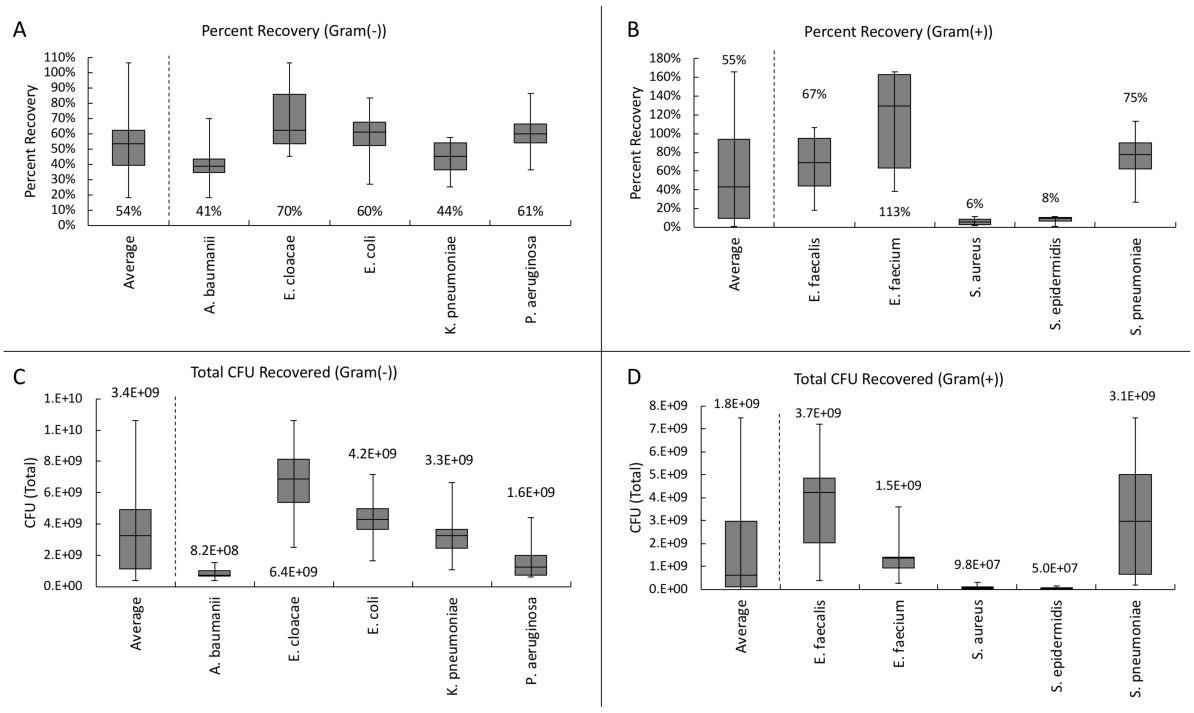


Figure 8. Bacteria recovery for a total of 83 strains of bacteria, with 49 Gram-negative (A and C) and 34 Gram-positive (B and D) organisms.

As seen in Figure 8, the average percent recovery for Gram-negative organisms was 54% (3.4 x 10^9 total CFU) and that for Gram-positive organisms was 55% (1.8 x 10^9 total CFU). The organisms with the least recovery out of the 10 groups of organisms tested were *S. aureus* and *S. epidermidis*, with less than 10% recovery and less than 10^8 total CFU recovered. Optimally, about 10^9 CFU is needed in the final output tube (for a 1 mL final volume) in order to obtain 10^6 CFU/µL to be spotted for MALDI.

Although the filter membranes used had low protein binding according to the manufacturer, some experiments suggested that there may be some loss of bacteria within the system due to adhesion of bacteria to surfaces. A PBC sample containing *S. aureus* was processed in four separate runs to observe the reproducibility of the filtration process. During filtration of the first sample, there was unexpected leakage in the filter,

so the retentate from that sample was recovered and a new filter was used for processing. Samples 2 and 3 were processed as normal using a new filter, but sample 4 reused the same filter used by sample 3 after a saline wash was done for that filter. Washing the filter used by sample 3 was believed to remove any bacteria remaining on the filter, but this assumption was proved to be false as shown in Figure 9. Here we incidentally have three conditions: (1) use of a new filter twice, for sample 1, (2) use of a new filter, for samples 2 and 3, and (3) reuse of an used filter, for sample 4. For samples 2 and 3 which were processed normally with a new filter, about 25% of the original number of bacteria in the PBC was recovered. However, 13% of bacteria in sample 1 was recovered using the new second filter and 40% of bacteria in sample 4 was recovered when reusing a filter. When performing a 2 sample t-test between samples 1 and 2, 1 and 3, 2 and 4, and 3 and 4, the p-values were <0.0001, 0.010, 0.003 and 0.003, respectively.

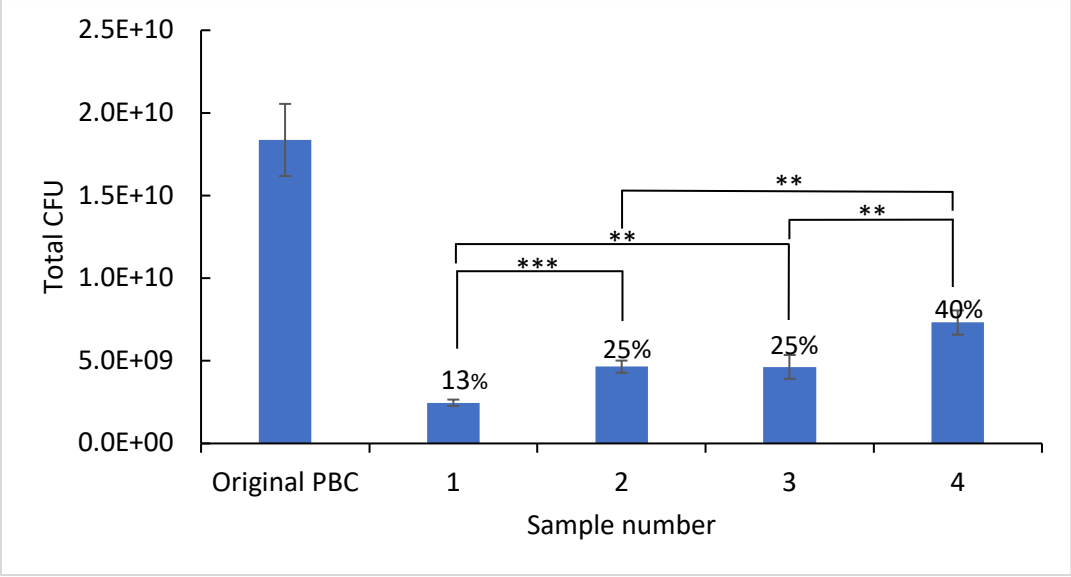
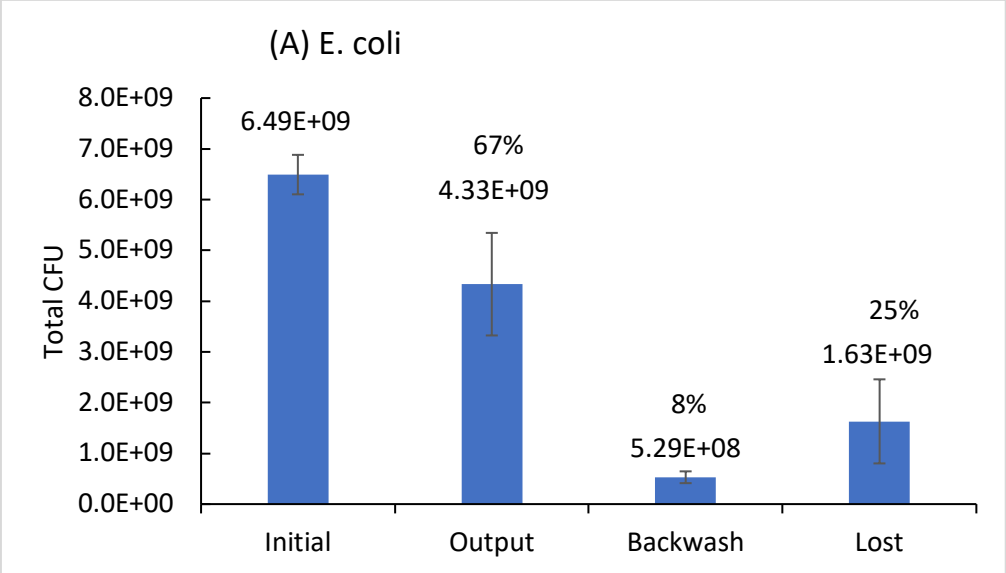


Figure 9. Recovery of S. aureus (29213) for 4 different samples to investigate filtration repeatability. Samples 2 and 3 used new filter membranes. Sample 1 used a new filter after the previous one was found faulty. The filter used for sample 3 was washed with saline prior to use by sample 4.

The different amounts of bacteria recovery from using filters of different conditions suggested that additional experiments were needed to investigate where bacteria loss occurred. Both Gram-negative (e.g. *E. coli*) and Gram-positive (*S. aureus*) organisms were tested to further understand areas of decreased bacteria recovery.



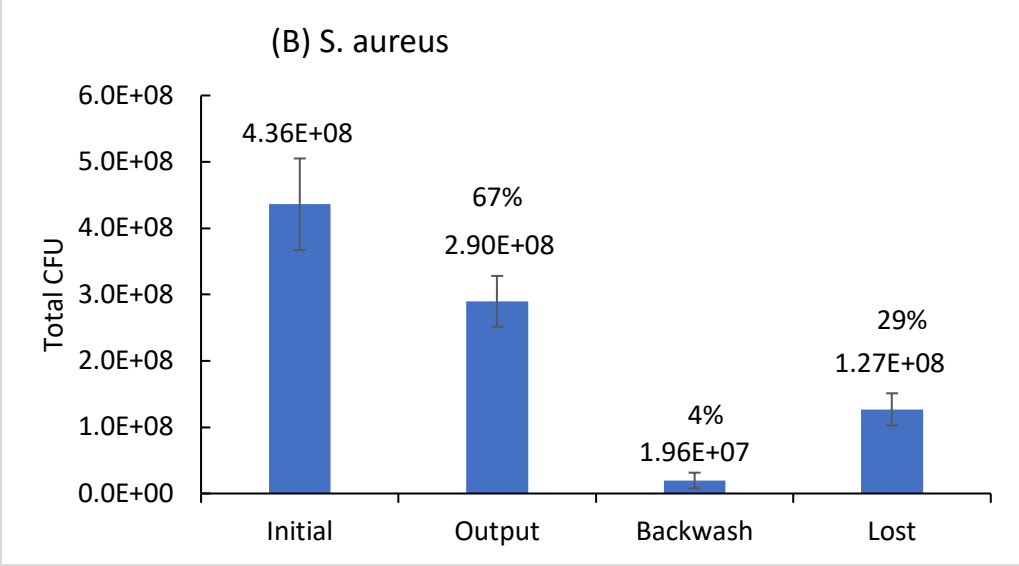


Figure 10. Recovery efficiency and loss for (A) *E. coli* and (B) *S. aureus*.

In Figures 10 A and B, the initial number of bacteria refers to the number of bacteria that was expected to be recovered in the final output. For the backwash sample, a small volume of saline (2 mL) was used to wash the filter membrane vigorously by pulsing the solution back and forth along the membrane. This step would potentially remove and recover any bacteria that may have reversibly attached to the membrane during the filtration process. In these two bacterial strains tested, there was about 25% of bacteria expected to be recovered that was lost for *E. coli* and 29% lost for *S. aureus*. Backwashing the filter expected to recover most of the bacteria remaining, but only a small fraction (4-8%) of the initial sample was recovered using that method. There was a strong possibility that bacteria were irreversibly bound to the filter membrane and hence could not be recovered by backwashing.

The above prediction could be confirmed by examining the membrane after filtration for any remaining bacteria. A 5 mL (0.72 McFarland) suspension of *E. coli* in saline was fluorescently stained with LIVE/DEAD BacLight Bacterial Viability Kit L7012 (Molecular Probes) by following the provided protocol. Then TFF was performed under normal processing conditions and the filter membrane was rinsed using saline solution to remove any bacteria on the surface that was not permanently attached. By using a fluorescent microscope, Figure 11 shows that a noticeable number of bacteria was adhered to the membrane. A great degree of bacterial adhesion occurred despite the fact that there were less bacteria in the initial sample compared to what is expected in an actual PBC. As seen in previous experiments, the amount of E. coli in a typical PBC is about 1.2×10^9 CFU/mL, which corresponds to a 4 McFarland sample.

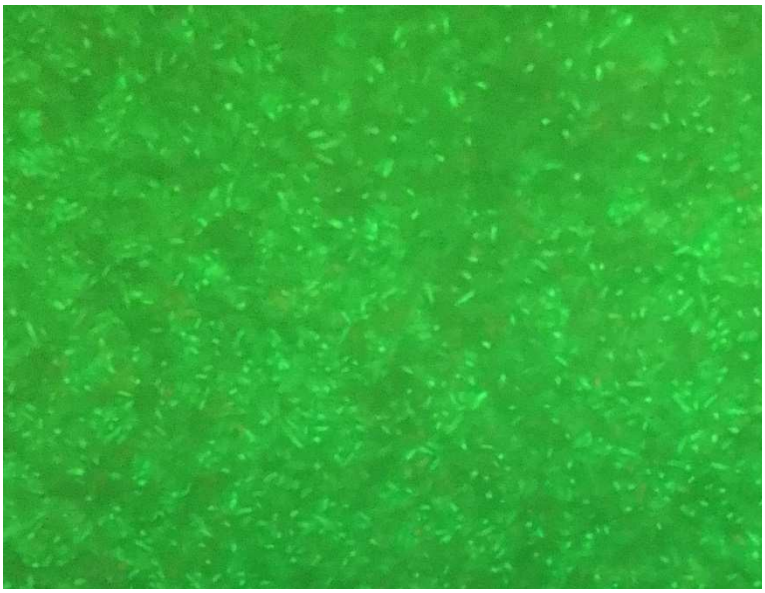


Figure 11. Fluorescently stained *E. coli* on a hydrophilic polycarbonate TEM

Mechanical processing of staphylococci samples prior to filtration

Staphylococci such as *S. aureus* and *S. epidermidis* were found to have the lowest recovery after filtration compared to other Gram-positive organisms tested. This was mainly attributed to their tendency to form grape like clusters that are comparable in size to blood cells; the large size of the bacteria would prevent them from filtering through the membrane's pores. Methods to attempt to break up the clusters into smaller units were tested to potentially increase recovery of these organisms. Chemical means such as the addition of detergents and physical means such as sonication did not show any apparent reduction in the size of the clusters.

The microfluidic device seen in Figure 12 was originally developed by an academic lab and evaluated by BD Technologies to break apart solid tumor tissue. This device was then investigated for this project to see if it could be translated over to break up bacteria clusters as well. The microfluidic device has chambers with progressively smaller channels and constrictions which create areas of high shear stress, allowing for

mechanical disruption to break apart clusters of bacteria. A syringe pump was initially employed to push and pull the sample across the device for twenty cycles before the standard filtration procedure using TFF was performed.

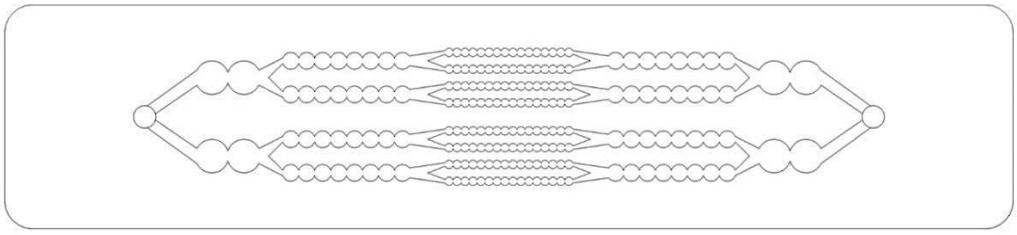


Figure 12. Microfluidic device evaluated to mechanically process and break up bacteria clusters.

PBC samples containing organisms *S. epidermidis* C2106 and *S. aureus* C2052 were processed using a syringe pump, whereas *S. aureus* 29213 was driven across the device using a vacuum pump. A vacuum pump was used in the latter sample to see if the PBC could be pulled across the channels at a faster speed, thus increasing the shear stress and potentially breaking up the staphylococci clusters into even smaller units. By employing the microfluidic device prior to standard filtration, the total number of bacteria recovered for organisms C2106, C2052, and 29213 increased by 156%, 132%, and 162% respectively, with p-values of 0.036, 0.013 and 0.083 from a paired t-test. The microfluidic device was effective in improving bacteria recovery for the staphylococci samples tested.

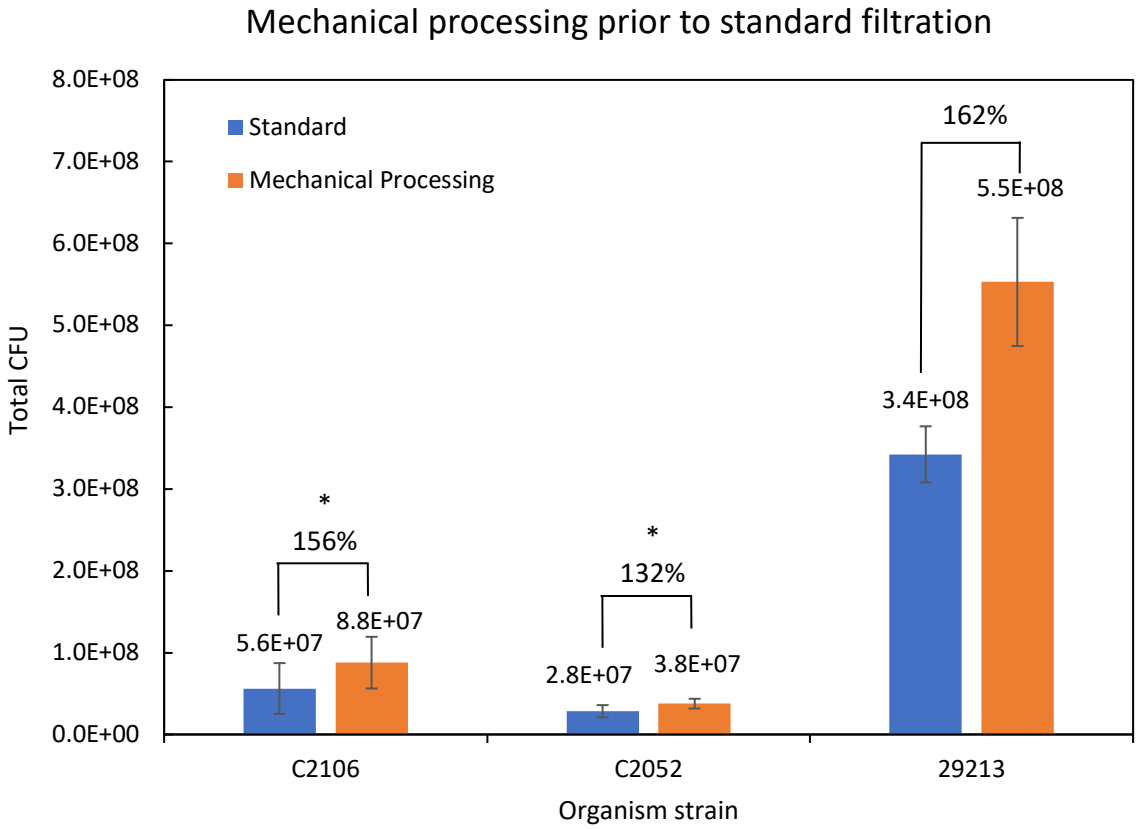


Figure 13. Comparison of bacteria recovery for Gram-positive organisms with and without mechanical processing prior to standard filtration with TFF. C2106 is *S. epidermidis*; C2052 and 29213 are *S. aureus*. (n=3 for each processing condition, per organism)

Recovery of bacteria at different times after positivity

One concern with the large bacteria recovery study was that such high recovery of bacteria was obtained from PBCs that were left in the BACTEC instrument to incubate for an additional 4-8 hours after positivity. Since the bacteria were allowed to grow further, these PBCs would have amounts of bacteria that were higher than they initially had when the bottle first went positive. Therefore, it might have been easier to recover a sufficient number of bacteria if there were more available initially. To investigate this potential issue, a study was designed to compare the recovery results from processing the

PBC sample at time zero when the bottle first went positive to that from processing the sample 6 hours after positivity. An aliquot of PBC was removed from the bottle at time zero and then the bottle was returned to the instrument to incubate for an additional six hours. We tested five strains each of Gram-negative and Gram-positive bacteria.

Table 4. Gram-negative bacteria recovery for processing PBCs at different times after positivity

		Total CFU recovered	
Organism	Strain	0 hr after positivity	6 hr after positivity
A. baumannii	19175	1.4E+09	1.0E+09
K. pneumoniae	11060	9.0E+08	6.0E+09
E. cloacae	17954	6.3E+08	4.0E+09
E. coli	11046	8.5E+08	4.1E+09
P. aeruginosa	C2006	1.4E+09	2.2E+09

Table 5. Gram-positive bacteria recovery for processing PBCs at different times after positivity

		Total CFU recovered	
Organism	Strain	0 hr after positivity	6 hr after positivity
S. pneumoniae	3993	2.0E+07	1.9E+09
S. epidermidis	3652	7.2E+08	1.2E+09
S. aureus	A29213	9.0E+07	2.6E+08
E. faecalis	A29212	1.1E+09	4.9E+09
E. faecium	A19434	1.6E+09	5.1E+08

The total amount of bacteria that was recovered immediately after the PBC went positive compared to 6 hours afterwards is shown in Tables 4 and 5. In all cases, as expected, there was at least equal or more bacteria in the original PBC after further incubation post positivity (data not shown). Accordingly, in all cases, there was equal or

more bacteria recovered in the PBCs processed 6 hours after positivity. For the Gram-negative organisms, samples processed soon after positivity recovered about 10^9 CFUs for each organism. However, for 2 of the 5 Gram-positive organisms tested, much less than 10^9 total CFUs were recovered and would therefore pose a problem for testing in MALDI ID.

Integration with Phoenix for antimicrobial susceptibility testing

The filtered bacteria sample needed to be tested to ensure that it was compatible with the preexisting BD Phoenix AST system. Microbial samples from the two bacteria isolation methods being compared were integrated with the BD Phoenix system for AST. The two methods were (1) reference method using subcultivation and (2) new PBC processing method using filtration. For this study, 39 Gram-negative, 35 Gram-positive, and 10 streptococcus pneumoniae organisms were tested, with about 50/50 mix of wild type and resistant strains of bacteria across the 3 types, and 43 unique antimicrobials tested. Table 6 summarizes the essential agreement and categorical agreement between the two methods for different drug-organism combinations and Table 7 shows the same data separated by organism. Essential agreement means that the MICs between the two methods compared are within ± 1 doubling dilution. Categorical agreement means that the susceptible, intermediate and resistant results between the two methods match. Overall, the essential and categorical agreement is greater than 90% for both Gram negative and Gram-positive bacteria, but there are a few drug-bacteria combinations for the Gram-positive organisms that match in less than 90% of cases for categorical agreement (data shown in appendix). Although that is the case, those few drug-bacteria combinations match in at least 85% of cases for categorical agreement.

Table 6. BD Phoenix AST results for bacteria isolates from traditional subcultivation compared to PBC processing by filtration

Panel Type	#Drug-Organism combinations tested	Essential Agreement	Categorical Agreement
Gram Negative	845	99.9%	97.9%
Gram Positive	699	99.0%	95.6%
Streptococcus	140	99.3%	95.7%

Table 7. BD Phoenix AST results for bacteria isolates from traditional subcultivation compared to PBC processing by filtration, separated by organism.

ORGANISM NAME	# OF STRAINS	TOTAL n =	ESSENTIAL AGREEMENT	CATEGORICAL AGREEMENT
Acinetobacter baumannii	8	127	99.2% (126)	96.9% (123)
Enterobacter cloacae	8	208	**100%**	99.0% (206)
Escherichia coli	8	213	**100%**	99.1% (211)
Klebsiella pneumoniae	8	205	**100%**	97.1% (199)
Pseudomonas aeruginosa	7	92	**100%**	95.7% (88)
ALL GN ORGS	**39**	**845**	**99.9% (844)**	**97.9% (827)**
Enterococcus faecalis	10	197	99.0% (195)	92.9% (183)
Enterococcus faecium	10	170	98.8% (168)	93.5% (159)
Staphylococcus aureus	9	207	98.6% (204)	97.6% (202)
Coagulase Negative Staph^	6	125	**100%**	99.2% (124)
ALL GP ORGS	**35**	**699**	**99.0% (692)**	**95.6% (668)**
Streptococcus pneumoniae	**10**	**140**	**99.3% (139)**	**95.7% (134)**
ALL ORGANISMS	**84**	**1684**	**99.5% (1675)**	**96.7% (1630)**

^ = 4 S. epi, 1 S. lug, 1 S. sap

ME S. epidermidis with P (1)

VME E. faecalis with TEC (2)

VME E. faecium with MXF (1) and P (1)

Integration with MALDI for bacteria identification

The potential to identify isolated bacteria from a filtered PBC using MALDI-TOF MS was evaluated in a preliminary study. For 11 different bacteria species, the processed output samples were prepared for MALDI using either the straight or diluted method and spotted two times onto the target plate, resulting in 44 total identifications.

The results outlined in Table 8 were based on the following interpretation of score values provided by the BioTyper software: score values ≥ 2.0 were regarded as correct identification at the species level, values between 1.7 and 1.99 were correct identification at the genus level, and score values < 1.7 were considered 'not reliable identification'. Using the new PBC processing approach, MALDI-TOF MS identified 70% of all bacteria at the species level and 16% at the genus level. For Gram-negative bacteria, 80% were correctly identified at the species level and 10% at the genus level. Of the Gram-positive bacteria, identification was correct in 63% of cases at the species level and 21% at the genus level. For bacteria prepared using the straight method, 64% were correctly identified at the species level and 18% at the genus level, but for those prepared using the diluted method, 82% were correctly identified at the species level and 14% at the genus level. There were no misidentifications observed for this set of bacteria studied. Overall, the identification BioTyper score was higher for Gram-negative bacteria compared with Gram-positive bacteria, and for the samples that were diluted compared to those tested straight from the output tube. As seen in Figure 14, there is a positive correlation between the bacteria amount in the sample size tested and the score produced by the MALDI—the more bacteria present, the more reliable the results. The diluted sample has half the amount of salt and bacteria as its straight tested counterpart. For the diluted samples, there are no samples that give "not reliable identification", but 18% of samples tested straight (4 out of 22) do, despite the fact that 3 out of 4 of those samples had enough bacteria spotted onto the target plate. From this preliminary data, a minimum bacteria amount of 6×10^5 is necessary to achieve a BioTyper score of ≥2 and the salt content in saline is not preferable for reliable results. The results show that these processed bacteria

samples were mostly appropriate for use with MALDI and able to provide at least genus level ID, but further studies are needed to determine what factors affect the ability to detect peaks in the instrument.

Table 8. Preliminary MALDI results for 5 Gram negative and 5 Gram positive bacteria. Note: MALDI ID in bold is a genus level ID (score < 2.00).

	Strain #	Reference ID	Method	MALDI ID (1)	MALDI ID (2)	Spotted sample (CFU/µL)
Gram negative organisms	9908	E. coli	Straight	E. coli	E. coli	5.0E+06
			Dilution	E. coli	E. coli	2.5E+06
	14780	K. pneumoniae	Straight	K. pneumoniae	K. pneumoniae	7.0E+06
			Dilution	K. pneumoniae	K. pneumoniae	3.5E+06
	9926	E. cloacae	Straight	E. cloacae	E. cloacae	3.8E+06
			Dilution	E. cloacae	E. cloacae	1.9E+06
	9915	P. aeruginosa	Straight	*no peaks found*	P. aeruginosa	1.5E+06
			Dilution	P. aeruginosa	P. aeruginosa	7.3E+05
	9928	A. baumannii	Straight	*no peaks found*	A. baumannii	1.2E+06
			Dilution	A. baumannii	A. baumannii	6.0E+05
Gram positive organisms	3073	E. faecalis	Straight	E. faecalis	E. faecalis	5.2E+06
			Dilution	E. faecalis	E. faecalis	2.6E+06
	C2101	S. saprophyticus	Straight	S. saprophyticus	*not reliable identification*	5.0E+05
			Dilution	S. saprophyticus	S. saprophyticus	2.5E+05
	7748	S. lugdunensis	Straight	S. lugdunensis	S. lugdunensis	1.4E+06
			Dilution	S. lugdunensis	S. lugdunensis	7.1E+05
	3945	S. pneumoniae	Straight	S. pneumoniae	*no peaks found*	1.1E+07
			Dilution	S. pneumoniae	S. pneumoniae	5.6E+06
	3993	S. pneumoniae	Straight	S. pneumoniae	*no peaks found*	7.5E+04
			Dilution	S. pneumoniae	*not reliable identification*	3.8E+04
	3998	S. pneumoniae	Straight	S. pneumoniae	S. pneumoniae	6.3E+06
			Dilution	S. pneumoniae	S. pneumoniae	3.1E+06

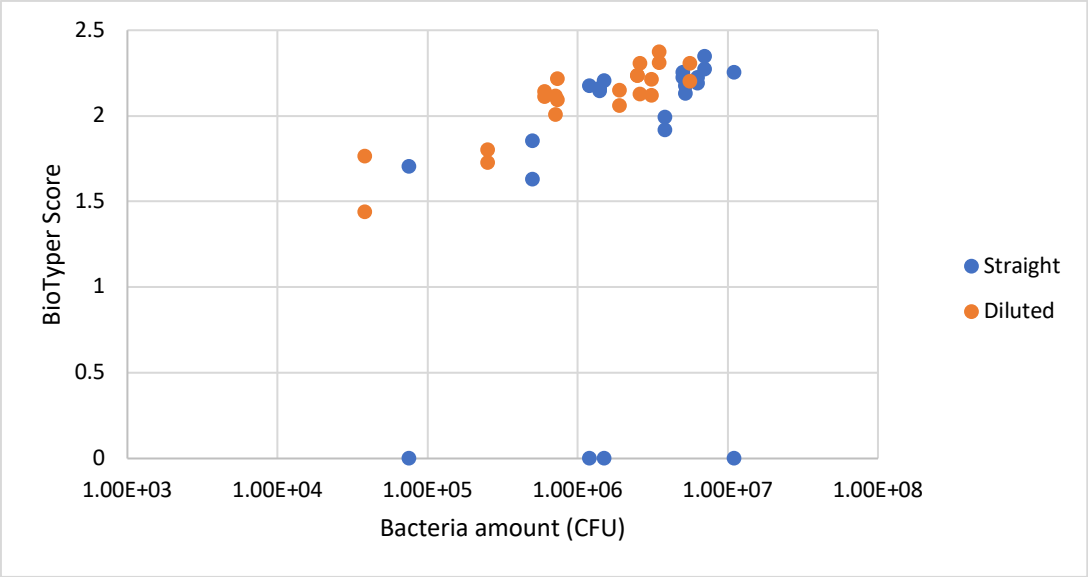


Figure 14. Correlation between bacteria amount spotted on the MALDI instrument and score produced

Discussion

The aim of this project was to develop and optimize a new method to isolate bacteria from a PBC and evaluate the efficacy in producing an output sample to be used with subsequent ID and AST.

Development of PBC processing method to isolate bacteria

The first part of this project focused on understanding the cellularity of the sample produced by the TFF system. During the early development of the PBC processing procedure, the final output sample usually had a pink tint in color due to any RBCs that had passed through the filter. The color itself was not a problem, but this indicated that any blood cells remaining would contribute to the turbidity measurements used to inoculate AST panels. If the blood cells contributed significantly to the turbidity measurements, then there would be less bacteria than expected in the inoculated AST tube, thus affecting the accuracy of the AST results.

As seen in Figure 6, the number of blood cells that passed through the 3 and 2 μm filters (about 10^7-10^8 CFUs) were found to be substantial compared to the total number of bacteria cells that were expected to be recovered (on the order of 10^7-10^9 CFUs). RBCs are widely understood to be deformable under certain flow conditions and thus were able to squeeze through the pores that are much smaller than the resting diameter of blood cells. The overlapping pores cannot be completely eliminated due to the inherent manufacturing process used to produce TEMs, but perhaps reducing the porosity of the membrane can reduce the likelihood of pores overlapping one another. Furthermore, diluting the feed sample during filtration can minimize cell-cell interaction and promote hydrodynamic lift, ensuring minimal contact of RBCs with the membrane surface.

Lysing the blood cells was a suitable option to address the presence of RBCs in the final sample. When RBCs are lysed, their cell contents are released, leaving behind empty membrane sacks, and the opaque suspension of blood cells becomes transparent. The data in Figure 7 suggests that adding lytic media in a 1:1 volumetric ratio to the processed sample is sufficient to reduce the contribution of RBCs to the turbidity measurement to a minimal amount. By the addition of this step, the inoculation of the Phoenix AST panels with a known bacteria concentration is more accurate, therefore leading to more reliable AST results as well.

Bacteria recovery and loss

This new bacteria isolation method using filtration needed to recover a minimum number of bacteria from PBCs to allow for use with ID and AST. There were no substantial problems regarding the recovery of the Gram-negative organisms tested in this study. However, less Gram-positive than Gram-negative bacteria were recovered from PBCs, resulting in inadequate amounts for downstream testing. Of the Gram-positive organisms tested, *E. faecalis, E. faecium* and *S. pneumoniae* exist in chains whereas *S. aureus* and *S. epidermidis* exist as clusters. The morphology of the latter two can explain why it is more difficult for those two organisms to be filtered compared to the others. The chains along their shorter dimension can still pass through the membrane's pores but sufficiently large clusters are unable to pass through.

Although adequate bacteria were recovered in the final output sample from most PBCs, the question of where the bacteria were lost to arose. As suggested with the study using filter membranes of different conditions, there was potentially some bacteria loss due to adhesion of bacteria to the filter membrane or left remaining in the dead space in

the system. As seen in Figure 9, the transfer of sample 1 to a new filter after the original one was found faulty resulted in a significant loss of bacteria compared to the expected recovery amount seen with samples 2 and 3. This was anticipated because there was dead volume in the filter and additional solution was not used to wash off and recover any remaining bacteria from the first filter before transferring to the next one. For sample 4, using a filter that was believed to be thoroughly washed before its second use resulted in higher recovery than usual. There are two potential reasons for this observation: (1) The bacteria from the previous sample was not completely washed away; there was some dead space in the filter where bacteria from sample 3 accumulated and that bacteria were eluted with the newly recovered sample 4 during the second time the filter was used. (2) Bacteria from the previous use of the filter may be irreversibly attached to binding sites on the filter and saturated them, so that new bacteria from the new sample cannot adhere to those surfaces. Therefore, there would be less bacteria loss and more recovery for the following sample. In respect to the first reason, the geometry of the filter may have crevices that bacteria want to settle in. Especially as the isolated bacteria is concentrated to produce the final output sample, each drop of liquid left in the system can have millions of bacteria. Backwashing with a small volume of liquid was used to retrieve any potential bacteria remaining on the filter membrane and in the system, but even with that additional step, there was still a significant number of bacteria not accounted for. This further supports the second reason that there were some bacteria that were irreversibly bound to the filter membrane or any other materials used in the system. In future studies, multiple backwashing steps may be useful in determining how many bacteria are

reversibly attached to surfaces in the system and can be recovered until only the permanently attached bacteria are left.

From the fluorescently stained bacteria sample in Figure 11, the image shows that substantial biofouling on the filter membrane occurred. However, this result may not be an accurate representation of how many bacteria would typically adhere to the membrane during processing. The cell suspension used had considerably less bacteria than what is typically found in a PBC and the degree of attachment may differ depending on the pore size of the filter. Also, a suspension of bacteria in saline was filtered rather than a PBC with blood cells and other components that may affect the degree of adhesion. Nevertheless, further optimization of the filter geometry and/or membrane properties is necessary to reduce this bacterial adhesion and subsequent loss. As mentioned previously, the choice of materials used and their surface characteristics can potentially help to reduce this attachment and should be considered further.

Another potential reason for less bacteria recovered than expected is the influence of high shear stress on microbial viability. There are studies that show that above a threshold value shear stress, the bacterial cells break, which is dependent not only on the microorganism species but also on the pump mechanism used to recirculate fluid during filtration. The viability loss was also influenced by the cellular concentration, number of passages through the shear stress device and exposure time. [40,41] Although this may not be likely as seen by promising AST results, confirmation by fluorescently staining the bacteria prior to filtration is possible to confirm the presence of dead cells in the final output sample.

Nature of initial PBC sample

The PBC processing approach was desired to be a universal method to process PBCs regardless of its initial condition. However, the results show that factors such as the original number of bacteria in the PBC and the type of bacteria present can affect recovery. Immediately after the blood culture bottle goes positive, there are less bacteria present compared to a sample that is allowed to incubate and grow further. In the current workflow, if a blood culture bottle goes positive in the middle of the night when there are no laboratory personnel working, then the bottle would be left in the incubator for additional time until it can be processed. However, it is beneficial for PBCs to be processed as soon as possible to achieve faster time to results. If the PBC processing method was part of an automated system, then this filtration method would need to recover enough bacteria from a blood culture that had just turned positive. For the Gram-negative organisms tested, there was sufficient bacteria recovered to perform further ID/AST testing even immediately after the bottle was flagged positive. However, some of the Gram-positive organisms had low and insufficient recovery from PBCs filtered shortly after positivity. To increase recovery, a mechanical method, such as the microfluidic device, can help break down Gram-positive bacteria clusters into smaller units prior to filtration so that they can be more readily extracted into the permeate. Another approach to increase the total bacteria recovered is to process a larger aliquot of PBC. The current method only uses 5 mL of PBC, so if recovery efficiency is similar for filtering larger volumes, more than enough bacteria should be recovered with up to 50 mL of PBC that can be used.

Integration with Phoenix AST and MALDI-TOF MS identification

The Phoenix AST results in Table 6 show that the new PBC processing approach produced an isolated and viable bacteria sample that yielded comparable results to the conventional approach of subcultivation. The high degree of agreement between the two methods suggests that the viability of the bacteria was not compromised significantly and there was minimal interference of blood background in the inoculation of the AST panels. For the few drug-organism combinations that don't match between the reference and experimental method, there could be a discrepancy between the number of bacteria that is expected in the 0.25 McFarland suspension and the amount that is actually inoculated due to the fact that turbidity measurements are estimations.

There are promising preliminary ID results using MALDI-TOF MS as shown in Table 8 and Figure 14. The suboptimal results can result from the presence of salt in the sample or insufficient number of bacteria spotted onto the MALDI target plate. MALDI is known to not prefer buffers, salts and detergents in the sample because it results in poor crystallization on the target plate and therefore interferes with the instrument readings.[42,43] Further studies are needed to replace the saline washing buffer with a different solution that will maintain the viability of the bacteria and not affect the results from MALDI. For this set of Gram negative bacteria, some samples such as *E. cloacae, P. aeruginosa* and *A. baumannii* produced better results after dilution, possibly due to the fact that the salt concentration was too high in the initial output sample. For the Gram-positive *S. saprophyticus* C2101 and *S. pneumoniae* 3993 strains, identification at the species level was not possible using the straight method nor with the diluted sample most probably due to too little bacteria present on the target plate. Especially for the *S. pneumoniae* 3993 sample, bacteria on the order of 10^4 CFU/µL was spotted onto the

target plate, which is drastically less than the 10^6 CFUs required for MALDI. One way to mitigate the risk of having too little bacteria is to layer multiple spots to achieve enough bacteria for a good signal. A much more detailed and thorough investigation into how these factors such as bacteria amount and salt concentration affect MALDI results is necessary to improve the quality of ID with bacteria isolates. Furthermore, testing samples isolated from the reference method in addition to those from the experimental method will allow for a deeper understanding to see if it was possibly a sample or bacterial strain issue that led to suboptimal results.

Proposed workflow

A modified workflow for the routine processing of PBCs is proposed in Figure 15. The initial Gram stain from a PBC will indicate if the sample is mono- or poly-microbial; if the sample is poly-microbial, then this new PBC processing approach cannot be used and the conventional subcultivation step is needed. Furthermore, the Gram-stain decides if a mechanical processing step prior to filtration is necessary; Gram-positive organisms should be mechanically processed to increase the likelihood of recovering more bacteria. As seen in Figure 1, *S. aureus* and Coagulase negative staphylococci are the second and third most common bacteria present in PBCs, comprising of 13.60% and 10.09% of all cases, respectively. Therefore, being able to recover suitable bacteria isolates from those PBCs is important due to their clinical prevalence. Then, after filtering the PBC sample and producing the bacteria isolate, ID and AST can be performed. Theoretically, by analyzing PBCs with this workflow, an ID result can be achieved on the day of positivity; the preliminary AST results can also potentially be obtained the same day, but the complete AST results will be obtained within 24 hours of

confirmation of sepsis by PBC. With this new PBC processing approach to isolate bacteria, diagnostic workflow and time to results is reduced by at least 12 to 24 hours.

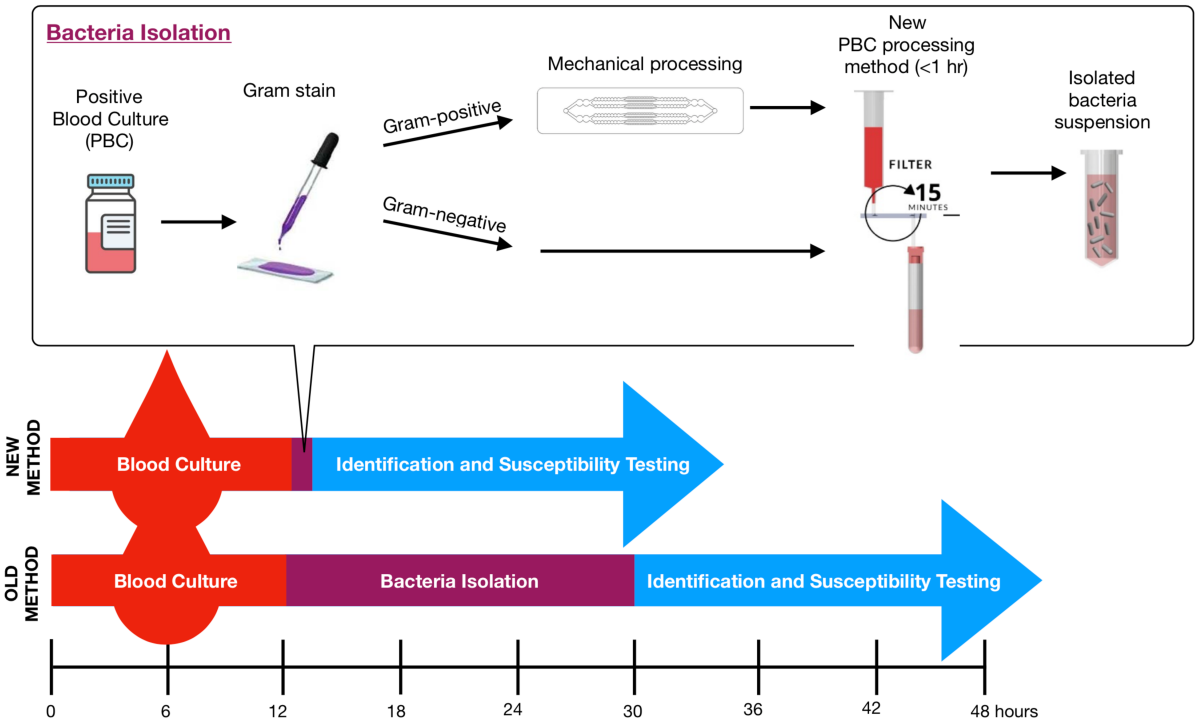


Figure 15: Modified workflow for processing of monomicrobial PBCs. With the new PBC processing method using TFF, time to results is reduced by on average 18 hours.

Conclusion

This project proposes to shorten time to results in sepsis diagnostics by replacing the conventional subcultivation step with a fast and automated approach. For clinical adoption, the proposed method needs to be able to produce an isolated bacteria sample that can be subsequently used for both ID and AST. Processes that are time consuming and require multiple manual preparations are not suitable for clinical applications. For a bacteria isolation method to be utilized in clinical settings, the method needs to handle a wide range of samples regardless of bacteria species present among other components in the PBC.

The proposed TFF based approach to isolate bacteria from a PBC is an effective solution to yield viable bacteria that can replace the subcultivation step and eliminate 12-24 hours in the PBC processing workflow. TFF is able to exclude at least 90% of blood cells from the isolated bacteria sample while recovering on average 3.4×10^9 total CFUs for Gram-negative organisms and 1.8×10^9 total CFUs for Gram-positive organisms. These viable bacteria isolates can be used for downstream testing since this method reduces the background that can contribute to inaccurate inoculation and results. This method is able to provide sufficient bacteria in most cases provided that workflow optimization is obtained. Direct testing of these bacteria isolates from blood culture gave a comparable performance for AST compared to the existing routine method of subcultivation. Based on the results of this study, the direct testing of filtered bacteria isolates with the BD Phoenix system could be confidently applied to AST of both Gram-negative and Gram-positive bacteria without reverting to subcultivation as an alternative. ID and AST results were available on average 18 hours sooner, which would allow for

quicker adjustment of antimicrobial therapy. Therefore, this alternative method to isolate bacteria from a PBC would help to expedite optimized and targeted antibiotic treatment after bacteria is identified.

As alluded to earlier, further design optimization of this filtration system is needed to improve performance. Although this method is able to capture sufficient Gram-negative bacteria, it is less effective in recovering Gram-positive organisms. For the various reasons that may result in reduced bacteria recovery, including the nature of the PBC sample and the adhesion of bacteria to system components, there are proposed methods to address these issues. For example, the microfluidic chip for the mechanical processing of PBCs is a promising technique to increase recovery of Gram-positive organisms. Also, the filter geometry, membrane properties and system parameters can be modified to maximize organism recovery and filtration efficiency. In addition to the biofouling factors previously mentioned, some studies suggest that the membrane roughness and stiffness can also affect cell adhesion, so those properties can be considered for the membrane material of choice. [34,37] Furthermore, the two main system parameters, flow rate and TMP, can be optimized to reduce caking and maintain permeate flux.

Compared to Bruker's Sepsityper solution, this filtration approach is simpler to use and yields a sample that is more versatile for subsequent testing. With the new method, the isolated bacteria sample from PBC is viable, so it can be used for not only ID but also AST. The TFF principle can be adopted for an automated instrument and eventually the entire process from incubating a blood culture to obtaining ID and AST results can be hands-free. Therefore, this less labor-intensive process with reduced hands

on time is appealing to lab technicians. The current filtration process takes about 15 minutes to complete, but any further reduction in time is beneficial since sepsis is a time-critical condition. By reducing the amount of time to isolate bacteria, the PBC processing method facilitates faster time to results for ID and AST, which enables improved antibiotic stewardship and results in positive clinical outcomes for septic patients.

Appendix

Table 1. Phoenix AST data for Gram negative bacteria-drug combinations

DRUG NAME	TOTAL n =	ESSENTIAL AGREEMENT	CATEGORICAL AGREEMENT
Amikacin	39	**100%**	97.4% (38)
Amoxicillin-Clavulanate	24	**100%**	95.8% (23)
Ampicillin-Sulbactam	32	**100%**	96.9% (31)
Aztreonam	31	**100%**	**100%**
Cefazolin	24	**100%**	**100%**
Cefepime	39	**100%**	97.4% (38)
Cefoxitin	24	**100%**	91.7% (22)
Ceftazidime	39	97.4% (38)	94.9% (37)
Ceftriaxone	32	**100%**	**100%**
Cefuroxime	24	**100%**	**100%**
Chloramphenicol	24	**100%**	**100%**
Ciprofloxacin	39	**100%**	97.4% (38)
Colistin	15	**100%**	**100%**
Ertapenem	24	**100%**	**100%**
Fosfomycin w/G6P	8	**100%**	**100%**
Gentamicin	39	**100%**	97.4% (38)
Imipenem	39	**100%**	97.4% (38)
Levofloxacin	39	**100%**	**100%**
Meropenem	39	**100%**	**100%**
Minocycline	32	**100%**	96.9% (31)
Moxifloxacin	18	**100%**	**100%**
Nitrofurantoin	24	**100%**	91.7% (22)
Norfloxacin	31	**100%**	**100%**
Piperacillin-Tazobactam	39	**100%**	97.4% (38)
Tetracycline	32	**100%**	93.8% (30)
Tigecycline	24	**100%**	**100%**
Tobramycin	39	**100%**	97.4% (38)
Trimethoprim-Sulfamethoxazole	32	**100%**	**100%**
ALL DRUGS	**845**	**99.9% (844)**	**97.9% (827)**

Table 2. Phoenix AST data for Gram positive bacteria-drug combinations

DRUG NAME	TOTAL n =	ESSENTIAL AGREEMENT	CATEGORICAL AGREEMENT
Ampicillin	20	**100%**	**100%**
Amikacin	15	**100%**	93.3% (14)
Chloramphenicol	35	**100%**	**100%**
Clindamycin	15	**100%**	**100%**
Ciprofloxacin	35	**100%**	85.7% (30)
Ceftaroline	9	**100%**	88.9% (8)
Daptomycin	34	**100%**	**100%**
Erythromycin	35	**100%**	94.3% (33)
Fosfomycin	10	**100%**	90.0% (1)
Nitrofurantoin	35	**100%**	88.6% (31)
Gentamicin	15	**100%**	**100%**
Gentamicin-Synergy	20	**100%**	**100%**
Levofloxacin	34	**100%**	97.1% (33)
Linezolid	35	**100%**	**100%**
Minocycline	35	**100%**	97.1% (34)
Mupirocin-High Level	15	**100%**	**100%**
Moxifloxacin	25	96.0% (24)	96.0% (24)
Norfloxacin	34	97.1% (33)	94.3% (33)
Oxacillin	15	**100%**	**100%**
Penicillin G	35	97.1% (34)	94.3% (33)
Rifampin	35	97.1% (34)	85.7% (30)
Streptomycin-Synergy	20	**100%**	**100%**
Trimethoprim/Sulfamethoxazole	15	**100%**	**100%**
Tetracycline	35	**100%**	94.3% (33)
Teicoplanin	34	94.1% (32)	94.3% (33)
Tigecycline	19	**100%**	**100%**
Vancomycin	35	97.1% (34)	97.1% (34)
ALL DRUGS	**699**	**99.0% (692)**	**95.6% (668)**

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Curriculum Vitae

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Education

Johns Hopkins University — Baltimore, MD
Master of Science in Materials Science and Engineering — Dec 2018
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Job Experience

Becton Dickinson — Sparks, MD
Systems Engineering Intern — Jan 2018-Present

- Collect and analyze data to support root cause analysis and troubleshoot hardware
- Collaborate within an interdisciplinary team to develop prototype instruments in product development
- Draft protocols and execute experiments to determine process capability and recommend improvements to software and/or hardware
- Contribute to Design Failure Mode and Effects Analysis (DFMEA) for a subsystem of the project to identify potential risks and risk mitigation solutions

Sepsis Innovation Co-op — Jun 2017-Jan 2018

- Participated in early-stage technology development research to generate new solutions to support pre-existing microbiology instruments
- Generated technologies and concepts to address business and customer needs
- Designed experiments to optimize and reduce to practice potential technical solutions
- Manufactured and evaluated prototype designs against requirements for downstream processes

Research Experience

Materials Science Department at JHU — Baltimore, MD
Research Assistant — May 2014-May 2017

- Synthesize polymeric gene delivery vehicles (nanoparticles) using Flash Nanocomplexation (FNC) with Professor Hai-Quan Mao, PhD
- Determine levels of cell uptake and gene expression by transfecting cancer cell lines with nanoparticles
- Gather and analyze data on the size and charge of the synthesized DNA-polymer complexes using Dynamic Light Scattering (DLS)
- Optimize Tangential Flow Filtration (TFF) system by troubleshooting different parameters
- Develop assay to quantify free polymer amount for nanoparticles and assess efficiency of TFF
- Purify nanoparticles using TFF in preparation for in-vivo studies in mice

Skills

Computer: Microsoft Word, Excel and PowerPoint; Matlab and Mathematica experience; Minitab
Laboratory: Gel electrophoresis; Cell culture; BCA assay; Luciferase assay; UV spectroscopy; Dynamic light scattering; Gel permeation chromatography; Tangential flow filtration; Flash Nanocomplexation; Optical Microscopy